FINAL

REPORT TITLE: Environmental Monitoring for Infectious Salmon Anemia Virus (ISAV) in and around Atlantic Salmon Marine Aquaculture Sites

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ABSTRACT

Seawater, cage pontoon and boat hull surfaces and blue mussels (*Mytilus edulis*) were evaluated for use in the detection of infectious salmon anemia virus (ISAV) at marine Atlantic salmon (*Salmo salar*) grow-out sites and as an early warning system to potential infection of fish and disease occurrence. Methods were developed and optimized for each sample type and implemented on field samples using cell culture and reverse transcription polymerase chain reaction (RTPCR) for ISAV detection. ISAV was consistently detected only by RTPCR in concentrated seawater samples collected up to 1.5 km from an affected site. Surface swab sample results correlated well with those from seawater, while mussels did not prove to be useful as potential sentinels in ISAV detection. Laboratory trials on ISAV longevity in seawater and freshwater showed that viability was directly correlated to temperature and biological activity, but the effect of temperature on detection by RTPCR was inversed in the two systems; while the longest period of ISAV detection by RTPCR in seawater was 11 weeks at 4°C, in freshwater this was 18 weeks at 16°C instead. Laboratory evaluation of mussels indicated that they did not significantly bioaccumulate ISAV from seawater and conversely may be destroying the virus through the filtration process. A quantitative RTPCR method was developed for ISAV and used to determine copy numbers in laboratory and field samples, and compare values to titers of viable virus determined through cell culture. All samples of fresh ISAV supernatant showed that a very high percentage of the virus detected by RTPCR was viable, while field samples indicated a 1000-fold difference. In ISAV longevity trials, despite the loss of viable virus early on ISAV copy numbers remained unchanged for many weeks, suggesting that a similar phenomenon is likely occurring in the field and that testing of concentrated seawater samples by RTPCR is probably the best method for the detection of ISAV shed into the environment.

EXECUTIVE SUMMARY

Environmental samples were evaluated for their potential use in the early detection of infectious salmon anemia virus (ISAV) in marine Atlantic salmon (*Salmo salar*) aquaculture sites before infection or disease occurrence. Methods were adapted and optimized for seawater, surface samples from cage pontoons and boat hulls, and blue mussels (*Mytilus edulis*) targeted for the testing. Assays to concentrate ISAV from seawater utilized charged filters and tangential flow filtration. Detection limity ranged from 10-100 viable virions per liter using the former with reverse transcription polymerase chain reaction (RTPCR) and <10 viable virions per liter by the latter method with both RTPCR and cell culture. Use of these assays to process seawater

samples from the field showed that ISAV could potentially be carried by tidal currents as far away as 1.5 km from infected sites, as detected by RTPCR. Results from surface samples correlated directly with those from seawater collected in parallel, but showed a lower level of sensitivity. Mussels collected from the field, even from sites that showed ISAV positive seawater, did not produce any ISAV positive results. Testing by cell culture indicated that viable virus was not present at detectable levels in any of the field samples.

Laboratory trials were conducted to determine the longevity of ISAV in seawater and freshwater. Results showed that degradation in seawater was directly correlated to temperature and biological activity with total loss of viability in as little as 24 hours in non-sterile treatments incubated at 16°C and longest survival in sterile treatments incubated at 4°C for up to 4 weeks. The decay in detection by RTPCR was not affected by incubation temperature, but correlated directly with biological activity; ISAV was detectable in sterile treatments at both temperatures for 11 weeks while detection was not possible after 4-5 weeks in the non-sterile treatments. ISAV viability in freshwater was also directly correlated with biological activity, but significantly lower than that in seawater; sterile treatments showed viability for 1-2 weeks while non-sterile treatments were non-viable after 1-4 days. However, an inverse correlation with incubation temperature was apparent particularly in RTPCR detectable virus; ISAV was not detectable in sterile samples at 4°C after 6 weeks while those incubated at 16°C showed detection for up to 18 weeks, and a similar trend was observed in non-sterile samples although detection ability disappeared earlier. The factor responsible for the longer-term preservation of RTPCR signal in treatments incubated at higher temperature is not yet determined.

The ability of mussels to bio-accumulate ISAV was tested in the laboratory. Results indicated that mussel filtration activity did not result in significant concentration of ISAV from seawater, and furthermore suggested degradation of the virus beyond that expected from incubation in seawater for the duration of the experiments.

A quantitative RTPCR (qRTPCR) method was developed for ISAV and optimized using known titers of virus. The assay was utilized to determine ISAV copy numbers in field samples and to compare virus viability versus RTPCR detection of genomic elements. Seawater and surface swab samples collected from ISAV-affected sites indicated the presence of 10^3-10^4 virus copies per liter of seawater or 100 cm^2 of surface area, but absence of any viable virus. Samples from *in vitro* assays however showed that, contrary to the hypothesis that a large proportion of ISAV produced is non-viable or defective, titers of viable ISAV in fresh supernatant preparations were equivalent to genome copy numbers determined by qRTPCR. ISAV longevity trials in seawater and freshwater indicated long-term conservation of the RTPCR signal despite the early loss of viability. Combined with the lack of viable ISAV detection in any of the environmental samples, collectively these results suggest that although a high percentage of the virions may actually be viable when first released into the natural environment, viability decreases rapidly in seawater and surfaces.

Overall, results indicate that testing by RTPCR of concentrated seawater samples may be the best method for detecting ISAV shed into the environment. This may however not always provide a preemptive clue to the infection of salmon at a particular site if the virus is spread by

other means such as introduction of infected fish or transmission of virus by sea lice (*Lepeophtheirus salmonis* or *Caligus spp.*).

PURPOSE

Problem / impediment of fishing industry addressed

This project addressed the specific priority listed in the NOAA/NMFS Notice for Solicitation for Applications published in the Federal Register Vol. 67, N0. 93, Section II, A. (Atlantic Salmon Aquaculture Development Considering the Endangered Species Status of Atlantic Salmon).

Due to concern that potential interaction with farm-raised Atlantic salmon (*Salmo salar*) through competition, interbreeding, and disease may threaten wild salmon populations in the Gulf of Maine, wild *S. salar* populations in eight Maine rivers, from the lower Kennebec River north to the US-Canada border, were listed as endangered in November 2000 under the Endangered Species Act (ESA) (16 USC 1531-1544). The listing has in turn placed the continuation of the Maine salmon aquaculture industry, the top producer of farm-raised salmon in the United States, in jeopardy. The loss of this industry would be a serious blow to the state's economy as well as to the increasing global demand for cultured finfish products. In order for salmon aquaculture to successfully continue in Maine, the negative impacts potentially imposed on endangered wild Atlantic salmon by farming activities, will need to be adressed.

Two issues of great concern to regulatory agencies referenced in the federal ESA listing proposal for Atlantic salmon were: 1) the appearance of disease in migrating wild salmon as a result of possible environmental pathogen loading by cultured salmon populations and, 2) the potential interaction of wild salmon with disease carrier escapees from aquaculture operations. This project focused on the first of these concerns. Farmed and wild salmon alike are at risk of being affected by many infectious agents and diseases. It is assumed that once infected with a pathogen, cultured fish stocked at high density at marine sites then pose a proportionally higher pathogen transmission risk to wild stocks. Development and improvement of diagnostic screening tools and surveillance programs for pathogens affecting salmon culture are essential not only for culture efficiency and economic viability of the industry, but also may help to minimize other or associated threats to natural resources such as wild Atlantic salmon.

The most significant disease currently affecting the cultured salmon industry in Maine is Infectious Salmon Anemia (ISA). The disease is induced by the viral agent infectious salmon anemia virus (ISAV) and shows variable infectivity and mortality affecting Atlantic salmon. ISA was first observed in Norway salmon aquaculture operations in 1984 (Thorud & Djupvik 1988), and subsequently reported from Scotland (Rodger et al. 1998, Rowley et al. 1999) among other European locations, as well as from the western North Atlantic, specifically New Brunswick, Canada (Mullins et al. 1998, Lovely et al. 1999, Bouchard et al. 1999, Blake et al. 1999,) and Maine, U.S.A. (Bouchard et al. 2001). To date, ISA has had a large negative economic impact on the salmon aquaculture industry in Maine through job losses and decreased revenues. The presence of this exotic pathogen and the disease experienced at Maine aquaculture sites led to the mandated destruction in late 2001 of more than 1 million Atlantic salmon raised at sites in Cobscook Bay, through a United States Department of Agriculture,

Animal Plant and Health Inspection Service (USDA, APHIS) sponsored ISA management program. This program, jointly administered through USDA, APHIS and the Maine Department of Marine Resources (DMR), provides an indemnity schedule that requires mandatory participation in biosecurity audits, site cleaning and disinfection protocols, integrated pest management, and the USDA, APHIS ISA surveillance program that monitors salmon stocks for ISAV at all active marine grow-out sites in Maine. The surveillance program focuses primarily on testing moribund salmon from production stocks in order to assess risk factors for ISAV transmission and to mitigate the prevalence of ISA disease through the rapid elimination of any marine cages testing positive for ISAV beyond thresholds established under the surveillance program. Micro Technologies, Inc. (MT) has been the principal diagnostic testing laboratory throughout the implementation of this program.

The causes and mechanisms of ISAV transmission and prevalence among populations at aquaculture sites are not fully understood. Wild fish populations may represent a reservoir for ISAV and vectors such as sea lice (*Lepeophtheirus salmonis* and *Caligus spp.*) may play an important role in its transmission. ISAV has been shown to be shed in mucus, reproductive fluids and feces from diseased fish before and after death, and may also be shed by infected but clinically inapparent fish. There is little information on how long ISAV can survive in the aquatic environment outside its host. Since the post-infection incubation period required for ISA disease expression may be between 12 and 42 days (Opitz, et al 1999), virus viability potentially plays an extremely important role in the overall infectivity thresholds needed to establish infection at a marine site. More basic research is needed to determine if, when, and under what conditions ISAV can be optimally detected in the environment, especially before it is detected in a salmon netpen population itself. In addition, it is important to know whether fish can become infected in freshwater, whether this plays a significant role in the infection cycle or conversely, whether freshwater can play a role in the clearing of ISAV from infected fish. Creation of a successful model for environmental monitoring to be utilized for early detection of important pathogens such as ISAV would offer a valuable management tool for improved husbandry techniques to reduce or eliminate disease outbreaks minimizing the risks of pathogen transmission to natural resources and wild Atlantic salmon.

Objectives

The overall aim of this project was to develop diagnostic methods for monitoring and surveillance of ISAV in the environment and to determine whether these could be used in an 'early warning system' to detect the virus before it could manifest itself in detectable levels in fish or cause a larger infection or full ISA/disease outbreak. The individual objectives were as follows:

1) To develop and optimize filter concentration and tangential flow assays for the detection of ISAV in seawater and freshwater.
2) To determine how long ISAV could remain viable and detectable in seawater and freshwater.
3) To develop and optimize methods for the detection of ISAV on surfaces such as cage pontoons and boat hulls.
4) To determine whether blue mussels, *Mytilus edulis*, often found attached to marine cages, can accumulate ISAV by filtration of seawater, and could possibly be used as an indicator species.

5) To compare the detection of viable ISAV trough cell culture versus viral RNA through RTPCR, and develop a quantitative RTPCR method in order to quantify the viral RNA present and ascertain the significance of positive findings as they related to pathogen presence.
6) To implement the methods developed in the field and determine their usefulness for monitoring Atlantic salmon grow-out sites for the presence of ISAV.

APPROACH

Description of work performed

The work consisted of a combination of laboratory and field testing performed by Micro Technologies, Inc. (MT) and Pisces Molecular LLC (PM). The milestones set for the project are listed below, followed by detailed descriptions of work involved toward the fulfillment of each milestone.

The laboratory work consisted of:
1) the optimization of a seawater filter concentration assay for ISAV and trials to determine the longevity of ISAV in seawater and freshwater (MT Milestone I),
2) optimization of diagnostic tools for testing surface samples and mussels (MT Milestone II),
3) mussel ISAV bioaccumulation trials (MT Milestone III),
4) the development of a quantitative RTPCR assay for ISAV (PM Milestones I, II and IV).

The field-work phase consisted of:
1) the monitoring of marine Atlantic salmon grow-out sites for the presence of ISAV using the optimized diagnostic assays for seawater, surfaces and shellfish (MT Milestone IV)
2) implementation of the quantitative RTPCR assay using field samples (MT Milestone V, PM Milestone III).

Seawater concentration assays

A preliminary protocol for concentrating ISAV from seawater and its detection through RTPCR was developed by MT (Giray et al. unpublished) before the commencement of this study by making modifications to methods already published for other viral agents (Abbaszadegan et al. 1999, Gilgen et al. 1997, Muscillo et al. 1997, McAllister et al. 1997, Maheshkumar et al. 1992, Maheshkumar et al. 1991). Before using this protocol in the project, the sensitivity of the assay and its ability to capture viable ISAV was determined. For this, two types of 47 mm filters, a positively charged MDS-1 Virosorb filter (CUNO Inc.) and a negatively charged 0.45 µm membrane filter (Millipore), were used to concentrate ISAV from seawater samples containing known titers of virus. The amount of viable virus recovered was determined through tissue culture infective dose 50% endpoint dilution assays ($TCID_{50}$) performed on chinook salmon embryo (CHSE-214) cell monolayers; 0.1 ml of each dilution was inoculated in quadruplicate onto wells in a 96-well plate. The amount of viral RNA recovered was determined using reverse transcription polymerase chain reaction (RTPCR) with the ISAV-specific 1D/2 primer set (Blake et. al. 1999) and the GeneAmp EZ rtTh amplification system (Applied Biosystems). Cell culture is the accepted 'gold standard' diagnostic tool for the detection of ISAV, but takes 2-4 weeks, depending on the cell line used, to show results. RTPCR is more sensitive and provides results

much sooner, but positive RTPCR results only indicate the presence of viral genomic material, and do not provide information on virus viability.

ISAV used to spike seawater samples during the following experiments was prepared by inoculation of 75 cm^2 flasks of CHSE-214 cells with ISAV and incubating at 16°C for 21 days. When cytopathic effect (CPE) due to ISAV was observed in 90% of each monolayer, supernatant from the flasks was harvested and pooled; cells were not scraped in order to reduce the possibility of introducing high-density pockets of virus into the experimental procedure. The combined virus stock was dispensed in 1 and 5 ml aliquots and stored in liquid nitrogen. Aliquots were used during the experiments and assumed to be 10^7 $TCID_{50}$ units per ml. All values given are based on this assumed titer. $TCID_{50}$ analysis was performed during each experimental trial on the stock virus dilutions, spiked seawater samples and eluates in order to determine exact titers of ISAV spikes and recoveries.

For the filter concentration assay sensitivity trials, 1-liter seawater samples at 4°C were pre-filtered through a glass fiber filter and the pH adjusted to 5.5. Ten-fold dilution series of ISAV supernatants were prepared from the previously frozen ISAV stock and these were then used to spike seawater samples in duplicate to produce series for the trials ranging from 10^0-10^7 $TCID_{50}$/ml. An aliquot was removed from each seawater preparation using a 3 ml syringe, filtered through a 0.45 µm filter into a sterile 5 ml tube and held at 4°C. Seawater preparations were immediately filter concentrated, starting with the preparation containing the lowest virus titer, using one of the two filter types described above and a vacuum filtration system. When using the CUNO Virosorb filters, two filters were stacked while only one filter was used with the membrane filter. The spiked seawater was passed through the filters at 25 ml per minute. Once the whole sample was filtered, the virus was eluted from the filter using two 7.5 ml aliquots of 3% beef extract (pH 10). The eluate and a 3 ml sample of the filtrate were filter sterilized using 0.45 µm syringe filters and held at 4°C. The filter was cut up into 2-3 mm wide strips and placed into 2 ml lysis buffer for extraction and testing by RTPCR. The efficiency of the filters to capture and elute virus was then determined through testing of the original preparation, filtrate, eluate and the filter by cell culture and RTPCR. The original ISAV dilution series used to spike samples was also tested by cell culture and RTPCR in order to determine initial titer of viable and RTPCR detectable virus. Due to production of cytotoxicity, undiluted seawater could not be placed directly on the cells, so the samples in the first wells were diluted 1:5 while the remainder followed serial 1:10 dilutions of the original sample. One ml aliquots of the supernatant dilution series, original preparation, filtrate and eluate were extracted for testing by RTPCR. Three trials were conducted for assay sensitivity determination.

The filtration assays were followed by the testing of a commercial tangential flow filtration system (Millipore Pellicon-XL and –Mini) in order to determine whether higher sensitivity could be obtained by the capability to increase the volume of seawater that could be filtered. The Pellicon-XL system was used to determine the optimum filter size that should be used in the assays in order to ensure the retention of ISAV while maximizing the sample volume capability and the speed with which samples could be processed. ISAV was spiked into pre-filtered seawater samples to produce 1 liter preparations containing virus at 10^0, 10^1, 10^2 and 10^3 $TCID_{50}$/ml. Starting with the lowest concentration, two 500 ml aliquots from each preparation were separately concentrated down to 15-25 ml. Samples were sterile filtered using 0.45 µm

syringe filters and along with a sub-sample of the original preparation and the filtrate, tested by cell culture and RTPCR as described above. The Pellicon-Mini system was then tested with the Biomax-100 and -300 filters in order to determine which would function better when filtering larger volumes (10-20 liters) of seawater.

ISAV Longevity in Seawater and Freshwater

Three laboratory trials were conducted in order to determine the longevity of ISAV in seawater. The first trial was performed as a pilot study to determine the rate of degradation of ISAV, the period for which the study would need to be carried out and to assess whether any problem areas in the design of the study needed to be addressed before a full-scale study was conducted. Six glass bottles each containing 250 ml of pre-filtered seawater were prepared and two of the bottles autoclaved. Previously frozen ISAV supernatant was filtered through a 0.45 μm filter and 2.5 ml of this stock was inoculated into each bottle to give a final 1:100 dilution of the stock. Bottles were mixed well and 2 ml immediately removed, filter sterilized using a 0.45 μm syringe filter and processed for testing by cell culture and RTPCR to determine initial titers. The salmon head kidney (SHK-1) and Atlantic salmon kidney (ASK) cell lines were used for $TCID_{50}$ determinations and RTPCR assays performed as before. One set of autoclaved and non-sterile bottles in duplicate were placed at 4°C while the second set was incubated at 16°C. Due to the components of the cell culture medium, a high level of bacterial growth occurred in the non-sterile bottles which in turn caused contamination problems in cell culture assays and the trial had to be terminated early. The trial was repeated by first centrifuging the ISAV stock through a 100 MWCO Centricon filter column in order to remove cell culture medium components. The concentrated sample was brought back up to the initial volume with PBS, filter sterilized and used for the inoculations. Samples were collected and processed as above at 1, 6 and 24 hours post-inoculation and then weekly for 6 weeks. A third trial was conducted using larger volumes of seawater and a longer incubation period. Four autoclaved and four non-sterile treatments of pre-filtered seawater were prepared and inoculated with ISAV to produce final titers of 10^4 $TCID_{50}$/ml. Sub-samples were immediately taken as before for testing by cell culture and RTPCR. Autoclaved and non-sterile containers were then incubated in duplicate at 4°C and 16°C. Samples were collected at 6 hours, 1, 2 and 4 days, and weekly for 13 weeks after inoculation and tested by cell culture and RTPCR, along with the original ISAV stock that was used for the inoculation.

The above trials were repeated to determine ISAV longevity in freshwater. For this, water was collected from Damariscotta Lake in Newcastle, Maine. Preliminary and larger-scale trials were conducted in the same manner as described for seawater.

Optimization of Tools for ISAV Detection in Surface Samples and Mussels

The optimization of cell culture and RTPCR assays for the detection of ISAV in surface samples and mussel tissues was accomplished by spiking RNA extracts, sample homogenates and raw samples with ISAV, followed by processing for testing by cell culture and RTPCR. The efficiency of recovering the spiked sample and the presence of inhibitors in the sample source were examined.

Sterile sponges (dimensions 5X3X2 cm) to be used for collecting samples from surfaces were spiked with known titers of ISAV in PBS, sliced into two, one half placed into 20 ml PBS and

the other into the same volume of 90% ethanol for testing by cell culture and RTPCR, respectively. The sponge half in PBS was processed by squeezing the supernatant into a homogenization bag, sterile filtering 3 ml of the sample through a 0.45 µm syringe filter and inoculating serial dilutions onto a 96-well cell culture plate containing CHSE-214, SHK-1 and ASK monolayers for $TCID_{50}$ analysis. The contents of the sponge half in ethanol was squeezed into the collection tube and 1 ml removed for RNA extraction and subsequent testing for ISAV by RTPCR. A dilution series of the RNA extracts from the original inoculum and that recovered from the sponge were compared in order to determine the efficiency of the extraction procedure and molecular assay sensitivity. In order to determine how organic materials in surface samples affects the assays, surface samples were collected from the field from known ISAV negative locations. One set of samples was spiked with known titers of ISAV and then processed for testing by cell culture and RTPCR as above. Another set was used to determine whether RTPCR inhibitors were present in the samples by extracting and spiking the RNA sample with known quantities of ISAV and comparing amplification results of dilution series prepared from the spiked samples versus ISAV stock used for the spike.

The optimization of testing methods for mussels involved the testing of several sample processing, homogenization, RNA extraction and amplification protocols in order to find the most practical and consistent method for producing accurate results. This proved more cumbersome than predicted. Initial amplifications performed on pooled mussel tissue homogenates spiked with ISAV indicated potential problems in both the RNA extraction and RTPCR amplification steps, and significant degradation of spiked virus or the presence of interfering agents. To address these issues, a combination of different extraction kits and protocols, parallel testing of ISAV spiked tissue RNA sample standards, variation of the RNA quantity used per reaction, and testing for inhibitors was performed.

The Qiagen Lipid Tissue Midi, the Qiagen RNeasy Mini and the Nucleospin RNA Extraction kits were compared for RNA extraction. Tissues were removed and pooled from several mussels, homogenized, and spiked with ISAV at high levels (10^5 $TCID_{50}$/ml homogenate) in order to establish the optimal extraction and amplification methods. These were followed by trials using a lower range (10^0-10^2 $TCID_{50}$/ml homogenate) of ISAV spikes to determine the sensitivity of the assay.

Mussel ISAV Bioaccumulation Trials

The ability of mussels to concentrate ISAV from seawater, the persistence of the virus in the tissues following filtration and the viability of ISAV after passage through mussels was investigated.

For the first trial, three 4-liter buckets each containing 3 liters of seawater were set up and maintained at ambient seawater temperature (8-10°C). Six mussels freshly collected from the field were placed in each bucket and allowed to acclimate. One treatment was used as a control while the remaining two were spiked with ISAV using previously frozen cell culture supernatant to obtain final titers of 10^3 and 10^4 $TCID_{50}$/ml. The mussels were allowed to filter for 2 hours and at the end of the filtration period, half of the mussels from each treatment were randomly removed, rinsed with seawater and placed into buckets of seawater not containing ISAV. The remaining half were rinsed with seawater to remove any ISAV on the shell surface, shucked, the

tissues pooled and homogenized using a hand-held laboratory homogenizer. Homogenates were processed for testing by cell culture and RTPCR. For cell culture testing, 600 µl of the tissue homogenate was diluted 1:50 in L-15, filter sterilized using a 0.45µm syringe filter, used to prepare serial dilutions from 10^0 to 10^{-5}, and 0.1 ml of each dilution inoculated in quadruplicate per well of a 96-well plate containing salmon head kidney (SHK-1) cell monolayers. For RTPCR 150 µl of the homogenate was extracted and used for amplification. In addition, initial and final titers of ISAV in the treatments and the supernatant stock were determined.

The second trial observed ISAV accumulation by mussels during a longer period of filtration and at lower ambient virus titers. Ten buckets each containing 3 liters of seawater were set up with aeration and maintained at ambient seawater temperature (8-10°C) throughout the experiment. The containers were inoculated in duplicate with ISAV at final titers of <1 to 10^3 $TCID_{50}$/ml using cell culture supernatant from a fresh virus culture. Water samples were taken immediately from each bucket to determine initial ISAV concentrations by cell culture and RTPCR. For the determination of viable virus titers by cell culture, seawater samples were diluted 1:5 in Leibovtz's L-15 medium (L-15), filter sterilized using a 0.45µm syringe filter and 0.1 ml inoculated in quadruplicate per well of a 96-well plate containing SHK-1 cell monolayers. RTPCR testing was achieved by extracting total RNA from a 100 µl sample using the Qiagen RNeasy kit and amplifying with the GeneAmp EZ rtTh system. Five mussels were then placed in each container and observed until their shells opened. In order to determine the level of ISAV detected in mussels before any filtration occurred, two mussels from each treatment were immediately removed, rinsed, homogenized in PBS using a hand-held unit and the homogenate used for testing by cell culture and RTPCR as described above to determine ISAV titers. The remaining three mussels in each treatment were allowed to filter seawater for 20 hours and then the individuals in each bucket removed, rinsed, shucked, whole tissues pooled and homogenized for ISAV detection and titer determination. Water samples were also taken and processed as above in order to determine remaining viable and RTPCR-detectable virus.

The above trial was repeated two more times to determine whether ISAV detected in mussels was actually virus retained in the tissues or attached to the tissue surfaces and further investigate the effect of feeding activity on the outcome. The experimental set-ups were similar to that above and testing was performed by cell culture and RTPCR at the start and end time points, which ranged from 4-24 hours, for the determination of viable and RTPCR-detectable ISAV in seawater and mussel tissues.

Field Testing

This portion of the project was commenced earlier than planned due to the detection of an ISAV-positive site that provided a perfect opportunity for testing the optimized tools in the field. The testing consisted of sample collection at designated ISAV-positive, ISA-clinical and ISAV negative sites in proximity to and at a distance from positive sites. Samples collected from each site were composed of 2-4 liter seawater samples from ISAV-positive and –negative cages and at increasing distance from the site, 100 cm^2 surface swabs from corresponding cage pontoons and boat hulls, sediment cores, and mussels attached to the cages. Kidney samples were also collected from Atlantic salmon in the same cages for testing as part of the USDA APHIS ISA Surveillance Program.

All environmental samples were collected and processed according to optimized methods described above, while kidney samples were processed and tested using standard operating procedures established at MT. Detection of both viable ISA virions and ISAV RNA was accomplished by the use of cell culture and RTPCR, respectively. In addition to the use of electronegative membrane filters for concentration of multiple low-volume (1-2 liters) seawater samples for testing by RTPCR, the tangential flow systems (Millipore Pellicon XL and Pellicon-2 Mini) were used for the concentration of larger volumes (10-20 liters) of seawater and for testing by cell culture as well as RTPCR.

Quantitative RTPCR Assay Development & Field Testing

The qRTPCR assay for ISAV was developed by PM. Two partially overlapping primer pairs with corresponding internal fluorogenic probes were designed from the ISAV segment 8 nucleotide sequence. Both primer & probe sets were tested in a qRTPCR assay and both sets amplified the expected size fragments from ISAV positive RNA samples and gave no signal with ISAV negative RNA samples. Results showed that the ISAV qRTPCR reaction for detecting ISAV with primer & probe set A was log linear across at least 6 orders of magnitude – from 10^7 molecules to 10 molecules, and had a threshold sensitivity of equal to or less than 10 molecules.

In order to ascertain agreement with RTPCR results and to determine ISAV genome copy numbers, the qRTPCR ISAV assay was tested with 51 known ISAV positive and negative sample RNAs from environmental samples and *in vitro* assays on longevity and bioaccumulation of ISAV. This was used to compare viable ISAV values obtained from the same samples in earlier testing by cell culture $TCID_{50}$ assays.

Project management

Project PI: Cem Giray, Ph.D.
Responsible for oversight of the project, development and optimization of the system for concentration of virus from water samples, planning and coordination of sample collection, design and implementation of experiments, supervision of PCR assays, evaluation and recording of results, preparation of all progress and final reports.

Co-PI: Deborah Bouchard
Supervision and performance of cell culture assays, assisting in planning and coordination of sample collection, assisting in experiments on ISAV longevity in freshwater.

Veterinarian: Peter Merrill, D.V.M.
Coordiantion and collection of field samples such as seawater, surface swabs and shellfish.

Assistants: Victoria Bowie, Keith Brockway, Brenda Maddox, Erica James
Preparation of cell culture assays, processing and testing of samples by cell culture and RTPCR, carrying out experiments designed with the PI and recording experimental results.

Subcontract: Pisces Molecular,LLC.
All aspects of the development of a quantitative RTPCR protocol for ISAV including the design and testing of fluorogenic probes, determination of reaction sensitivity and testing of environmental samples using the assay.

FINDINGS

Seawater concentration assays

The results for two trials conducted using the electropositive MDS-1 Virosorb filters are given in Tables 1 and 2. The use of these filters for the detection of ISAV in spiked seawater samples, although producing somewhat promising results in the first trial, overall did not produce the expected level of virus concentration and retrieval. It was determined that the RTPCR and cell culture assays used in the study could detect ISAV at concentrations of 1-10 and 50-100 viable virus particles per ml, respectively. After filter concentration, ISAV was detected by RTPCR in seawater samples spiked with 10^3-10^4 viable virions per liter (equivalent to 1-10/ml) but not in any of the lower spiked concentrations. Testing by cell culture also revealed that significant sample concentration did not occur, cytopathic effect (CPE) being observable only in seawater samples spiked at $10^{1.5}$-10^2 $TCID_{50}$/ml. Further testing indicated that a significant portion of the virus was passing through the filter rather than being captured and could be detected in the filtrate even in seawater samples spiked at concentrations of 10 viable virions per ml. However, the direct extraction of filters for RTPCR after elution did produce detection in some samples spiked at 100 virions per liter. Since only 1 ml of the total 15 ml volume of eluent was extracted, a minimum 10-fold and possibly 100-fold concentration had to occur for RTPCR detection which showed sensitivity down to 1-10 virions in testing of known supernatant dilutions. However, bands viewed in gel electrophoresis were weak and results between replicate samples inconsistent. Testing performed using electronegative membrane filters (Millipore) showed similar findings (Table 3). Extraction of filters after elution and testing by RTPCR did indicate 10-100-fold concentration of the sample. This was again not observed in the eluates, suggesting that virus could not be eluted sufficiently from the filter. The failure of both filters to release upon elution ISAV that was concentrated from seawater samples may have been due to the ionic composition of seawater which may affect the dynamics of virus capture and release by the filters. Until improvements can be made to the assay with additional experimentation on elution efficiency, testing for viable virus by cell culture can be only performed on samples containing ISAV at titers of 10^2 $TCID_{50}$/ml and above, but direct testing of filters by RTPCR can provide detection of ISAV in seawater at 10-100 virions per liter.

Tangential flow filtration systems produced the expected concentration of ISAV from all spiked preparations. Using the Pellicon XL system, it was determined that the 100,000 MW cut-off filter (Biomax 100) yielded the best concentration of pre-filtered seawater samples, but was significantly slower in sample processing than the 300,00 MW cut-off filter (Biomax 300). The use of the latter, although faster, caused loss of virus through the filter, sometimes resulting in up to one log lower sensitivity than the Biomax 100. However, when raw seawater was filter concentrated, the Biomax-100 clogged easily and resulted in a prohibitive build-up of pressure. It was concluded that even though some virus loss occurred with the Biomax-300 filters, the gain in processing time and the ability to process more seawater in less time may offset the loss. The tangential flow filtration system showed 100% success in concentrating RNA genome and viable ISAV as determined by RTPCR and cell culture, respectively. No reduction in virus viability was observed as a result of the filter concentration process.

Table 1. Trial 1 - Seawater filter concentration using MDS-1 Virosorb filters.

Virus stock			Spiked Seawater			Eluate		
Assumed conc. (virus/ml)	**RTPCR**	**$TCID_{50}$/ml**	**Expected conc. (virus/ml)**	**RTPCR**	**$TCID_{50}$/ml**	**Expected conc. (virus/ml)**	**RTPCR**	**$TCID_{50}$/ml**
10^7	pos	$1X10^{6.75}$	$10^{4.75}$	pos	$1X10^{4.25}$	$10^{5.45}$	pos	$1X10^{4.25}$
			$10^{4.75}$	pos	$1X10^{4.0}$	$10^{5.45}$	pos	$1X10^{4.0}$
10^6	pos	$1X10^{5.75}$	nt	nt	nt	$10^{4.45}$	pos	$1X10^{3.25}$
			nt	nt	nt	$10^{4.45}$	pos	$1X10^{3.0}$
10^5	pos	$1X10^{4.25}$	$10^{2.25}$	pos	$1X10^{1.75}$	$10^{2.95}$	pos	$1X10^{2.75}$
			$10^{2.25}$	pos	$1X10^{2.0}$	$10^{2.95}$	pos	$1X10^{2.50}$
10^4	pos	$1X10^{3.0}$	nt	nt	nt	$10^{1.7}$	pos	$1X10^{2.25}$
			nt	nt	nt	$10^{1.7}$	pos	no CPE
10^3	pos	$1X10^{2.5}$	$10^{0.5}$	pos	no CPE	16	pos	no CPE
			$10^{0.5}$	pos	no CPE	16	pos	no CPE
10^2	pos	$1X10^{1.5}$	nt	nt	nt	2	neg	no CPE
			nt	nt	nt	2	neg	no CPE

Table 2. Trial 2 - Seawater filter concentration using MDS-1 Virosorb filters.

Virus stock			Spiked Seawater		Eluate			Filtrate	Filter
Assumed conc. (vir/ml)	**RTPCR**	**$TCID_{50}$/ml**	**Assumed conc. (vir/lt)**	**RTPCR**	**Expected conc. (vir/ml)**	**RTPCR**	**$TCID_{50}$/ml**	**RTPCR**	**RTPCR**
10^7	pos	$1X10^6$	nt	nt	nt	nt	nt	nt	nt
10^5	pos	$1X10^{5.5}$	10^5	pos	$1X10^{4.2}$	pos	$1X10^{2.75}$	pos	pos
			10^5	pos	$1X10^{4.2}$	pos	$1X10^{1.25}$	pos	pos
10^4	pos	$1X10^{3.75}$	10^4	pos	$1X10^{2.45}$	pos	no CPE	pos	pos
			10^4	pos	$1X10^{2.45}$	pos	no CPE	pos	pos
10^3	pos	$1X10^{2.75}$	10^3	neg	28	neg	no CPE	neg	neg
			10^3	neg	28	neg	no CPE	neg	pos
10^2	pos	$1X10^{1.75}$	10^2	neg	3	neg	no CPE	neg	neg
			10^2	neg	3	neg	no CPE	neg	pos
10^1	neg	no CPE	10^1	neg	<1	neg	no CPE	neg	neg
			10^1	neg	<1	neg	no CPE	neg	neg

Table 3. Trial 3 - Seawater filter concentration using electronegative membrane filters. (nt = not tested).

Virus stock			Eluate			Filter	
Assumed conc. (virus/ml)	**RTPCR**	**$TCID_{50}$/ml**	**Expected conc. (virus/ml)**	**RTPCR**	**$TCID_{50}$/ml**	**Initial conc. (virus/lt)**	**RTPCR**
10^6	pos	$1X10^{5.25}$	nt	nt	nt	nt	nt
10^5	pos	$1X10^5$	$1X10^{3.82}$	pos	$1X10^{2.75}$	10^5	pos
			$1X10^{3.82}$	pos	$1X10^{1.75}$	10^5	pos
10^4	pos	$1X10^{3.5}$	$1X10^{2.32}$	pos	No CPE	$10^{3.5}$	pos
			$1X10^{2.32}$	neg	No CPE	$10^{3.5}$	pos
10^3	pos	$1X10^{2.5}$	$1X10^{1.32}$	neg	No CPE	$10^{2.5}$	pos
			$1X10^{1.32}$	neg	No CPE	$10^{32.5}$	pos
10^2	pos	$1X10^{1.5}$	2	neg	No CPE	$10^{1.5}$	pos
			2	neg	No CPE	$10^{1.5}$	pos
10^1	pos	No CPE	nt	nt	nt	nt	nt
10^0	pos	No CPE	nt	nt	nt	nt	nt

ISAV Longevity in Seawater and Freshwater

The testing showed that ISAV viability decreased rapidly when incubated at 16°C in non-sterile seawater, but could persist up to 4 weeks when incubated in sterile seawater at 4°C (Figure 1). ISAV was detected by RTPCR for up to 11 weeks in sterile seawater maintained at both temperatures, but was undetectable in non-sterile treatments after 4-5 weeks incubation (Table 4). Considering that $1/100^{th}$ of the extracted RNA (equivalent to 10 µl of initial sample if extraction is 100% efficient) is used in the amplification reaction and after a 1:5 dilution an equivalent of 20 µl of the original sample is inoculated in the first set of wells for TCID50 assays, neither method had a significant advantage over the other in these trials. Results therefore indicate that although ISAV may be detected in samples by RTPCR, a large portion of the sample is likely genomic material that belongs to non-viable virus.

The results of experiments on the survival of ISAV in freshwater were similar to those of ISAV longevity in seawater except that the ISAV titers in all treatments at the start of the experiment were lower, at approximately $10^{3.4}$ $TCID_{50}$/ml, which may have been the reason for comparatively earlier loss of viable virus. Testing by cell culture showed ISAV CPE in sterile treatments for over 1 week incubation at both temperatures with no viable virus detected after two weeks (Figure 2). Loss of viability was faster in the non-sterile treatments, but contrary to the trend in seawater, disappeared more rapidly in samples incubated at 4°C (ISAV CPE not observed after 24 hours) than those incubated at 16°C (ISAV CPE observed for four days).

Table 4. ISAV longevity in seawater RTPCR results (shown as positive/negative for replicates).

TIME	4°C Sterile	4°C Non-sterile	16°C Sterile	16°C Non-sterile
0 hrs.	++	++	++	++
6 hrs.	++	++	++	++
24 hrs.	++	++	++	++
48 hrs.	++	++	++	+-
96 hrs.	++	++	++	+-
1 week	++	++	++	+-
2 weeks	++	+-	++	+-
3 weeks	++	+-	++	--
4 weeks	++	+-	++	+-
5 weeks	++	+-	++	--
6 weeks	++	--	++	--
7 weeks	++	--	++	--
8 weeks	++	--	++	--
10 weeks	+-	--	+-	--
11 weeks	+-	--	+-	--
13 weeks	--	--	--	--

Figure 1. ISAV longevity in seawater - Cell culture results (avg. values presented for each treatment).

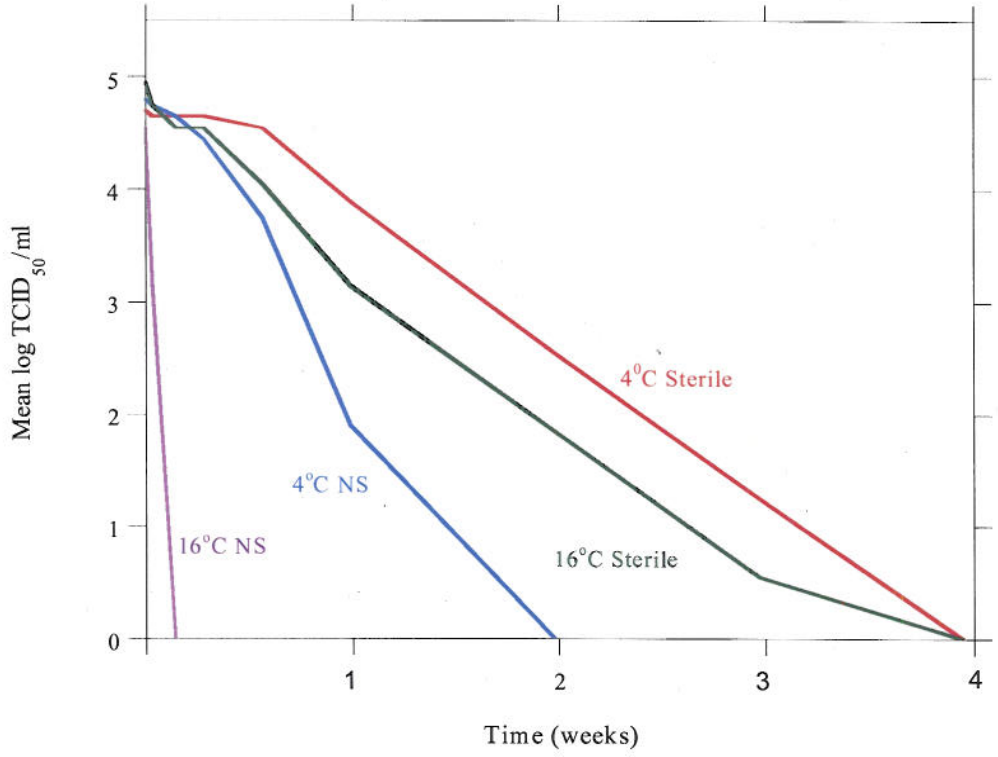
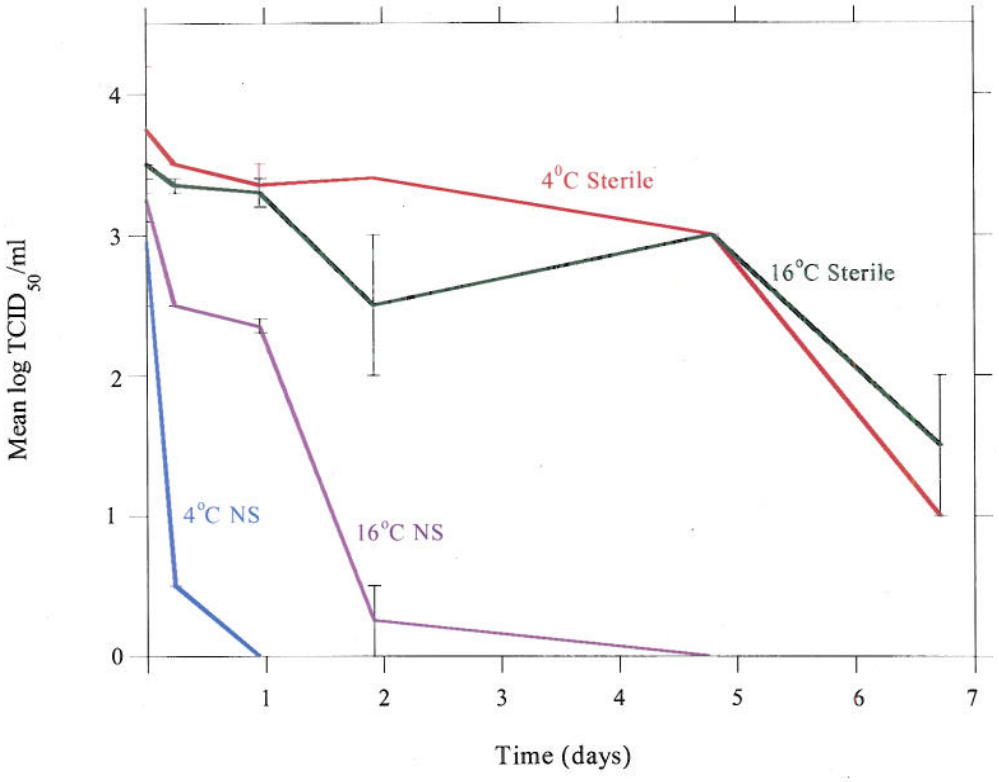


Table 5. ISAV longevity in freshwater RTPCR results (RTPCR positive/negative for replicates).

TIME	4°C Sterile	4°C Non-sterile	16°C Sterile	16°C Non-sterile
0 hrs.	++	++	++	++
6 hrs.	++	++	++	++
24 hrs.	++	++	++	++
48 hrs.	++	++	++	++
120 hrs.	++	++	++	++
1 week	++	--	++	++
2 weeks	++	--	++	++
3 weeks	++	--	++	+-
4 weeks	++	--	++	--
5 weeks	++	--	++	--
6 weeks	+-		++	--
7 weeks	--		++	
8 weeks	--		++	
9 weeks	--		++	
11 weeks			++	
12 weeks			++	
13 weeks			++	
15 weeks			++	
16 weeks			++	
17 weeks			--	
18 weeks			+-	
19 weeks			--	

In similar fashion, ISAV was detected by RTPCR in sterile treatments incubated at 4°C for 6 weeks while detection continued for 16-18 weeks in sterile treatments incubated at 16°C (Table 5). Detection by RTPCR faded out faster in non-sterile treatments, but as in the sterile treatments, detection lasted several weeks longer in treatments incubated at 16°C; 1 week versus 3-4 weeks. Furthermore, despite the ISAV titer inoculated into freshwater treatments being 1 log lower than that for seawater trials, ISAV was detectable for a significantly longer period in freshwater preparations incubated at 16°C than seawater preparations at both temperatures. The reason for this anomaly is not yet determined. As far as the difference observed between seawater and freshwater trials, it is possible that ionic constituents and slightly higher pH of seawater may cause earlier degradation of the virus. The difference in degradation rate of both viability and genomic elements is however difficult to explain.

Figure 2. ISAV longevity in freshwater - Cell culture results (avg. values presented for each treatment).



Optimization of Tools for ISAV Detection in Surface Samples and Mussels
Protocols as described above for the processing of surface samples worked extremely well. ISAV was detected in sponge samples spiked at 10 virions per ml, within the detection limit of the RTPCR assay.

The best results for extraction of mussel tissues were obtained by using the Qiagen RNeasy RNA extraction kit. RTPCR amplifications required a 1:100 dilution of the RNA extract in order to obtain clean, accurate and consistent reactions. Using this methodology, ISAV inoculated into mussel tissues at 10^2 $TCID_{50}$/ml of homogenate, was detected. Comparison of ISAV spiked mussel RNA extracts, tissue homogenates and PBS controls indicated that the extraction and amplification was not affected by any inhibitors that may be present in the tissues.

Mussel (*Mytilus edulis*) ISAV Bioaccumulation Trials
Results indicated that ISAV could be detected in the tissues of mussels incubated for as little as 1 hour in seawater containing ISAV titers of 10^3-10^4 $TCID_{50}$/ml. However, trials conducted for longer periods using a 10-fold dilution series, and in some cases with phytoplankton added to induce feeding, indicated that no significant concentration of the virus was occurring. Instead, the lack of an increase in virus titer in the tissues, and observations of a decrease in ISAV titer in

water samples beyond that would be expected normally as shown in the ISAV longevity trials, may indicate actual destruction of virus during passage through the mussels. None of the trials showed the presence of viable virus in mussels by cell culture testing performed following the incubations. Additional work is being conducted to examine the issue that the virus may actually be destroyed rather than concentrated in mussel tissues.

Field Testing

Field testing was performed ahead of schedule during December 2003-February 2004 as a result of the detection of a new ISAV strain variant in Maine during routine sampling for the USDA ISA Surveillance Program and access to a confirmed ISA-positive site that was being harvested. The assays developed for testing field samples produced successful results in the detection of the new ISAV strain variant in seawater samples which correlated well with testing results from affected salmon, but surface samples and mussels collected from the same site did not produce ISAV-positive results. The confirmed ISA-positive site showed ISAV in multiple seawater and surface swab samples, but no significant virus detection in mussels; only one mussel showed a weak positive result by RTPCR which may have been due to ISAV contaminating the surface of the tissue from the seawater itself. ISAV was detected by RTPCR in seawater as far away as 1.5 km from ISAV-positive sites, indicating that the virus could potentially be spread by seawater from sites experiencing outbreaks. In addition, well-boat and boat hull surface swabs showed that ISAV was being carried between sites by boat traffic and expulsion of well-boat contents. The results of environmental testing performed are summarized in Table 6, some of which was made possible by this grant while the remainder was conducted using funds from other sources.

Table 6. Summarized results of environmental samples tested.

SAMPLE	Total # tested	# ISAV/RTPCR +	# ISAV CPE +
Seawater	273	58	0
Cage pontoons	91	7	0
Boat hulls	36	6	0
Mussels	53	1 (weak +)	0
Atlantic salmon	285	170	49 of 58 tested

Additional field work was conducted in Summer and Fall 2004 at an ISAV emerging site, an adjacent ISAV-negative site. No ISAV-positive results were obtained from any of the samples by RTPCR or cell culture. ISAV was only detected in the fish sampled from the site at the same time for the USDA ISA Surveillance Program. The lack of detection of ISAV in environmental samples may have been due to the new management system implemented as a result of biosecurity measures used at the sites following earlier work performed in the field on the presence of ISAV in seawater and other sample sources. These measures, which included the removal of affected cages before adjacent cages can be infected with ISAV, prohibition of the release of well-boat contents into the surrounding waters and controlling boat traffic between

sites, could in turn have accounted for a significant decrease in the likelihood of detecting ISAV in the environment.

Quantitative RTPCR Assay Development & Field Testing

The assay was successfully developed and preliminary tests conducted by PM. Samples containing known concentrations of viable virus, and extracts from *in vitro* studies on ISAV longevity and mussel ISAV bioaccumulation, and environmental samples were submitted to PM by MT for quantification of ISAV copies in each sample by qRTPCR.

Results showed that in dilution series controls of ISAV prepared in cell culture media, spiked extracts of seawater and mussel RNA, spiked raw seawater and freshwater, and homogenates of mussels incubated in seawater, the amount of viable ISAV, as determined by $TCID_{50}$ assays, was very close to the number of genome copies detected by qRTPCR. This is contrary to suggestions that only a small proportion of virus detected by RTPCR, as low as $1/1000^{th}$, is viable. The fact that all of these samples were prepared by using cell culture supernatant may be an important factor in the results showing even numbers of viable and RTPCR detectable virus.

All environmental samples tested that were positive for ISAV by RTPCR, were found to not contain any detectable viable virus. Similar levels of ISAV were found in all of these samples, ranging from 10^3 to 10^4 virion genome copies per sample (2 liters seawater or 100 cm^2 swab equivalents). Another interesting observation was obtained from the freshwater ISAV longevity trials, where it was determined through qRTPCR that the same amount of ISAV was present in a sterile treatment incubated at 16°C after 18 weeks incubation as was initially inoculated, but viable virus had not been detectable one week after the initiation of the experiment. It appears that although the virus lost viability or the ability to infect fish cell lines soon after inoculation into freshwater, the genomic elements did not show any significant degradation during the 18 weeks, likely aided by the sterile conditions in the experimental treatment. Although degradation of genomic material was faster in non-sterile treatments, viability still disappeared significantly sooner than RTPCR detectable elements. These observations from field and *in vitro* samples may provide support for expecting low viability versus copy number in samples obtained from the natural environment.

EVALUATION

Project goals and objectives

MT milestones I-V and PM milestones I-IV have been accomplished. Two filter concentration methods, using charged filters and tangential flow filtration, were developed and tested for use in the detection of ISAV in seawater samples. Although the filter concentration assays provided detection of ISAV by RTPCR at relatively high sensitivities, the capture and release of viable virus was not as successful. Additional effort will be expended to address inefficiencies in the elution step. Tangential flow filtration produced the expected results for both viable and RTPCR detectable virus, and filled this gap for the purpose of this project. Diagnostic tools for the detection of ISAV in surface and blue mussel (*M. edulis*) samples were successfully developed and produced good results both in laboratory trials and in field samples. Trials to investigate the ability of mussels to bioaccumulate ISAV sufficiently showed that this was not a good sample

source to test for early detection of the virus. Field testing provided an abundant number of positive environmental samples in earlier trials due to the presence of multiple ISA-positive and ISAV infected sites. This allowed the testing of all assays, and showed that seawater and surface swabs were good sample sources for the detection of ISAV in and near infected sites. The qRTPCR assay was successfully developed by PM and tested on both laboratory and field samples. Results matched very well to those obtained for the same samples by routine RTPCR and comparisons of cell culture versus RTPCR detectable virus were accomplished, providing valuable insight into the proportion of viable virus that could be expected in ISAV-positive laboratory and field samples.

An extension was requested until December 2004 in order to complete the mussel infectivity trials and field testing. No modifications were made to the goals and objectives. The preparation of the final report was delayed in order to further investigate some of the findings and ensure that the final manuscript submitted provided sufficient conclusions.

Dissemination of Results

The work performed has been presented at several forums:

The 2nd Maine Atlantic Salmon Technical Advisory Committee Research Forum, 7 January 2004, University of Maine, Orono, Maine.

29th Annual Eastern Fish Health Workshop, 22-26 March 2004, Atlantic Beach, North Carolina.

In addition, findings have been included in several related presentations by others to refer to the work conducted.

The work and findings will be prepared for publication in scientific journals as three separate manuscripts; the filter concentration and surface swab sampling methods and their use in the field, ISAV longevity in seawater and freshwater, and the effect of mussel bio-filtration on ISAV viability, respectively, incorporating qRTPCR results into each part.

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